Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis

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**Activation of the cell surface receptor Fas/APO-1
(CD95) induces apoptosis in lymphocytes and regulates
immune responses. The cytoplasmic membrane protein
Bcl-2 inhibits lymphocyte killing by diverse cytotoxic
agents, but we found it provided little protection against
Fas/APO-1-transduced apoptosis in B lymphoid cell
lines, thymocytes and activated T cells. In contrast, the
cowpox virus protease inhibitor CrmA blocked Fas/
APO-1-transduced apoptosis, but did not affect cell
death induced by γ-radiation or serum deprivation.
Signalling through Fas/APO-1 did not down-regulate
Bcl-2 or induce its antagonists Bax and Bcl-x_S. In Fas/
APO-1-deficient lpr mice, Bcl-2 transgenes markedly
augmented the survival of antigen-activated T cells
and the abnormal accumulation of lymphocytes
(although they did not interfere with deletion of auto-
reactive cells in the thymus). These data raise the
possibility that Bcl-2 and Fas/APO-1 regulate distinct
pathways to lymphocyte apoptosis.**
Keywords: apoptosis/APO-1/*bcl-2*/CrmA/Fas/lymphocytes

Introduction

The proper functioning of the immune system depends
upon mechanisms ensuring that only appropriate lympho-
cytes survive. Whether a lymphocyte lives or dies is
largely determined by signals relayed through cell surface
receptors. Some of these, including many cytokine recep-
tors, signal survival while others, such as Fas/APO-1,
signal death. Many lymphocytes die during the early
stages of their development, probably because they fail to
receive a survival signal via antigen receptors ('death by
neglect') or because they bear receptors that react with
self antigens ('activation-induced death'). At the end of
an immune response, most activated lymphocytes also
die, again due to either the absence of a survival signal
or the activation of a killing signal. All of these cell deaths
occur by apoptosis, a common physiological process
for removing unwanted cells, recognizable by chromatin
condensation, cytoplasmic shrinkage and DNA degrada-
tion (Wyllie *et al.*, 1980; Ellis *et al.*, 1991).

Some insight into the molecular mechanisms controlling
cell death has derived from genetic studies on the nematode
Caenorhabditis elegans. Cells fated to die during the
development of this organism do so only if they express
two genes, *ced-3* and *ced-4* (Ellis *et al.*, 1991). While no
vertebrate homologue of *ced-4* has yet been identified,
ced-3 encodes a protein homologous to interleukin-1β-
converting enzyme (ICE), a cytoplasmic cysteine protease
(Thornberry *et al.*, 1992; Miura *et al.*, 1993; Yuan *et al.*,
1993). This enzyme and a family of related cysteine
proteases (Kumar, 1995) can provoke apoptosis in certain
cell types and are implicated as natural death effectors in
vertebrate cells (Gagliardini *et al.*, 1994; Lazebnik *et al.*,
1994; Enari *et al.*, 1995; Los *et al.*, 1995).

The first physiological inhibitor of cell death to be
identified (Vaux *et al.*, 1988) was Bcl-2, the cytoplasmic
membrane-associated product of an oncogene activated by
a chromosome translocation in human follicular lymphoma
(Korsmeyer, 1995). It is homologous to Ced-9, which
inhibits programmed cell death in *C.elegans* (Vaux *et al.*,
1992; Hengartner and Horvitz, 1994a), and to several
mammalian proteins. One of these, Bcl-x_L, also promotes
cell survival (Boise *et al.*, 1993), but others such as Bax,
Bcl-x_S (a splice variant of Bcl-x_L), Bad and Bak antagonize
the survival function of Bcl-2 and Bcl-x_L (Boise *et al.*,
1993; Chittenden *et al.*, 1995; Farrow *et al.*, 1995; Kiefer
et al., 1995; Yang *et al.*, 1995). How the proteins of this
family regulate cell death remains obscure, but Bcl-2 and
Bcl-x_L are potent inhibitors of apoptosis induced under
various conditions of cell stress, including growth factor
deprivation, irradiation, and treatment with glucocorti-
coids, phorbol ester, calcium ionophores or cytotoxic drugs
(Vaux *et al.*, 1988; Sentman *et al.*, 1991; Strasser *et al.*,
1991). The broad spectrum of protection suggests that
Bcl-2 inhibits a common effector pathway, but the bio-
chemical mechanism is unknown. Although Ced-9 can
prevent all programmed cell deaths in *C.elegans*, Bcl-2
does not block all physiological forms of death in lympho-
cytes, since *bcl-2* transgene expression has only minimal
effects on the deletion of autoreactive B and T cells
(Sentman *et al.*, 1991; Strasser *et al.*, 1991, 1994; Hartley
et al., 1993; Nisitani *et al.*, 1993). This suggests that there
are multiple pathways to death subject to distinct regulation
(Strasser, 1995).

Fas/APO-1 (or CD95) is a cell surface protein that
induces apoptosis via activation of an ICE/Ced-3-related
protease (Enari *et al.*, 1995; Los *et al.*, 1995; Tewari and
Dixit, 1995). A member of the tumour necrosis factor
(TNF) receptor family, Fas/APO-1 was discovered as the
target of antibodies cytolytic for certain tumour cells
(Trauth *et al.*, 1989; Yonehara *et al.*, 1989; Itoh *et al.*,
1991; Oehm *et al.*, 1992). Its natural ligand, a member of
the family of TNF-like cytokines, is primarily expressed
on the cell surface but also found in soluble form (Suda
et al., 1993). Engagement of the T cell receptor (TCR)
on T hybridoma cells or activated normal T cells induces
expression of both Fas/APO-1 and its ligand, resulting in

autocrine or paracrine activation of apoptosis (Russell and Wang, 1993; Russell et al., 1993; Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). The spontaneous mouse mutations lpr and gld are loss-of-function alleles of the genes encoding Fas/APO-1 and its ligand, respectively (Watanabe-Fukunaga et al., 1992; Lynch et al., 1994; Takahashi et al., 1994). The characteristic lymphadeno-pathy and auto-immune syndromes that develop in these mice (Cohen and Eisenberg, 1993) indicate that the receptor and its ligand play an important role in regulating lymphocyte death. There is strong evidence that Fas/APO-1 is involved in the limitation of immune responses and in T cell-mediated cytotoxicity (Russell and Wang, 1993; Russell et al., 1993; Kägi et al., 1994; Lowin et al., 1994), but it is not essential for the apoptotic death of autoreactive T cells in the thymus (Sidman et al., 1992; Herron et al., 1993).

We demonstrate here that, within one cell line, Bcl-2 inhibits γ-radiation-induced but not Fas/APO-1-induced apoptosis, while the protease inhibitor CrmA inhibits Fas/APO-1-induced but not γ-radiation-induced cell death. Moreover, in the lymphoid system of bcl-2 transgenic lpr mice, gain of Bcl-2 plus loss of Fas/APO-1 synergize to promote lymphadenopathy and the survival of activated T cells. These data provide evidence that Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis.

Results

Bcl-2 does not inhibit apoptosis induced by Fas/APO-1 signalling
We first investigated the impact of Bcl-2 on apoptosis transduced via Fas/APO-1 in cells of the human B lympho-blastoid line SKW6 and the mouse B lymphoma line CH1. Cells transfected with a retroviral vector co-expressing a human bcl-2 cDNA and a neomycin resistance gene (bcl-2/neo) were selected in G418 and assayed for Fas/APO-1 and Bcl-2 protein expression by flow cytometric analysis of cell surface and cytoplasmic immunofluorescence, respectively. Several independent clones expressing parental levels of Fas/APO-1 and high levels of Bcl-2 (e.g. Figure 1A and B) were selected for subsequent analysis.

Cells of the SKW6 line readily undergo apoptosis when incubated with a monoclonal antibody (mAb) specific for human Fas/APO-1 (Trauth et al., 1989). Their loss of viability was not significantly changed by high levels of Bcl-2. The sensitivity of SKW6 bcl-2/neo cells over a range of concentrations of antibody was unaltered and their death was only marginally slowed (Figure 1C). Similarly, CH1 bcl-2/neo cells remained sensitive to induc-tion of apoptosis by an antibody to mouse Fas/APO-1, even though Bcl-2 had rendered them resistant to γ-irradiation (Figure 1D).

The failure of Bcl-2 to prevent Fas/APO-1-transduced killing in these cell lines might be related in some way to their malignant phenotype. We therefore investigated whether Bcl-2 could protect untransformed lymphocytes from Fas/APO-1-induced apoptosis using T cells from bcl-2-25 transgenic mice (Strasser et al., 1991). Like thymocytes from normal mice (Ogasawara et al., 1993), most thymocytes from bcl-2 mice expressed Fas/APO-1 (Figure 2A). Anti-Fas/APO-1 antibody accelerated the death of normal thymocytes, as shown previously

(Ogasawara et al., 1993). It also accelerated the death of thymocytes overexpressing Bcl-2, but had no effect on thymocytes from lpr mice (Figure 2B). (The higher overall viability of the antibody-treated bcl-2 thymocytes can be accounted for by the fact that Bcl-2 reduces the spon-taneous death of thymocytes in tissue culture.)

Similar results were obtained with activated T cells. Stimulation with mitogens plus interleukin-2 (IL-2) induces Fas/APO-1 expression on T cells from normal and bcl-2-25 mice but not those from mutant lpr mice (Figure 2C). Subsequent reactivation of T lymphoblasts via their antigen receptor or with phorbol ester plus ionomycin induces expression of the Fas/APO-1 ligand, causing their death by apoptosis (Figure 2D; Russell and Wang, 1993; Russell et al., 1993; Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). In this system, the presence of IL-2 in the cultures obviates spontaneous death due to lack of cytokines, allowing the effect of Fas/APO-1 to be dissociated from the effect of cytokine deprivation. It was readily apparent that activated T cells from bcl-2 mice were as sensitive as those from normal mice while, as expected (Russell et al., 1993), cells from lpr mice were totally resistant (Figure 2D).

Taken together, these results establish that high levels of Bcl-2 do not effectively inhibit the death process initiated in lymphocytes by activating Fas/APO-1 with agonistic antibodies or the physiologic ligand.

Fas/APO-1 signalling does not alter expression of Bcl-2, Bax or Bcl-x
In view of the efficacy of Bcl-2 against so many other cytotoxic stimuli, its failure to block Fas/APO-1-induced apoptosis was surprising. Might Fas/APO-1 signalling inhibit Bcl-2 action? There was no evidence of decreased synthesis or enhanced degradation of Bcl-2 since SKW6 and CH1 cells contained similar levels of endogenous and transgene-encoded Bcl-2 protein before and after treatment with Fas/APO-1-specific antibodies (Figure 3A, and data not shown). Furthermore, the levels of two inhibitors of Bcl-2, Bax and Bcl-x$_S$, were unchanged as assessed by Western blotting and cytoplasmic immunofluorescence staining, and by reverse transcriptase-PCR analysis, respectively (Figure 3B and C, and data not shown). In addition, the pattern of proteins that co-immunoprecipita-ted with Bcl-2 was the same in untreated and anti-Fas/APO-1 antibody-stimulated SKW6 cells (data not shown). These results do not exclude the possibility that Fas/APO-1 signalling might interfere with Bcl-2 function by other mechanisms, for example by post-translational modification of Bcl-2 or Bax, or by inducing other inhibitors. Nonetheless, they raise the possibility that Fas/APO-1 signalling triggers death by a mechanism that bypasses Bcl-2.

CrmA inhibits Fas/APO-1-induced but not γ-radiation-induced apoptosis
The cysteine protease Ced-3 is essential for all pro-grammed cell deaths in C.elegans (Ellis et al., 1991; Miura et al., 1993; Yuan et al., 1993), but mammalian cells express several cysteine proteases which might participate selectively in the execution of different cell death stimuli. To test this possibility we studied the effects of the cowpox virus-encoded protein CrmA, a potent

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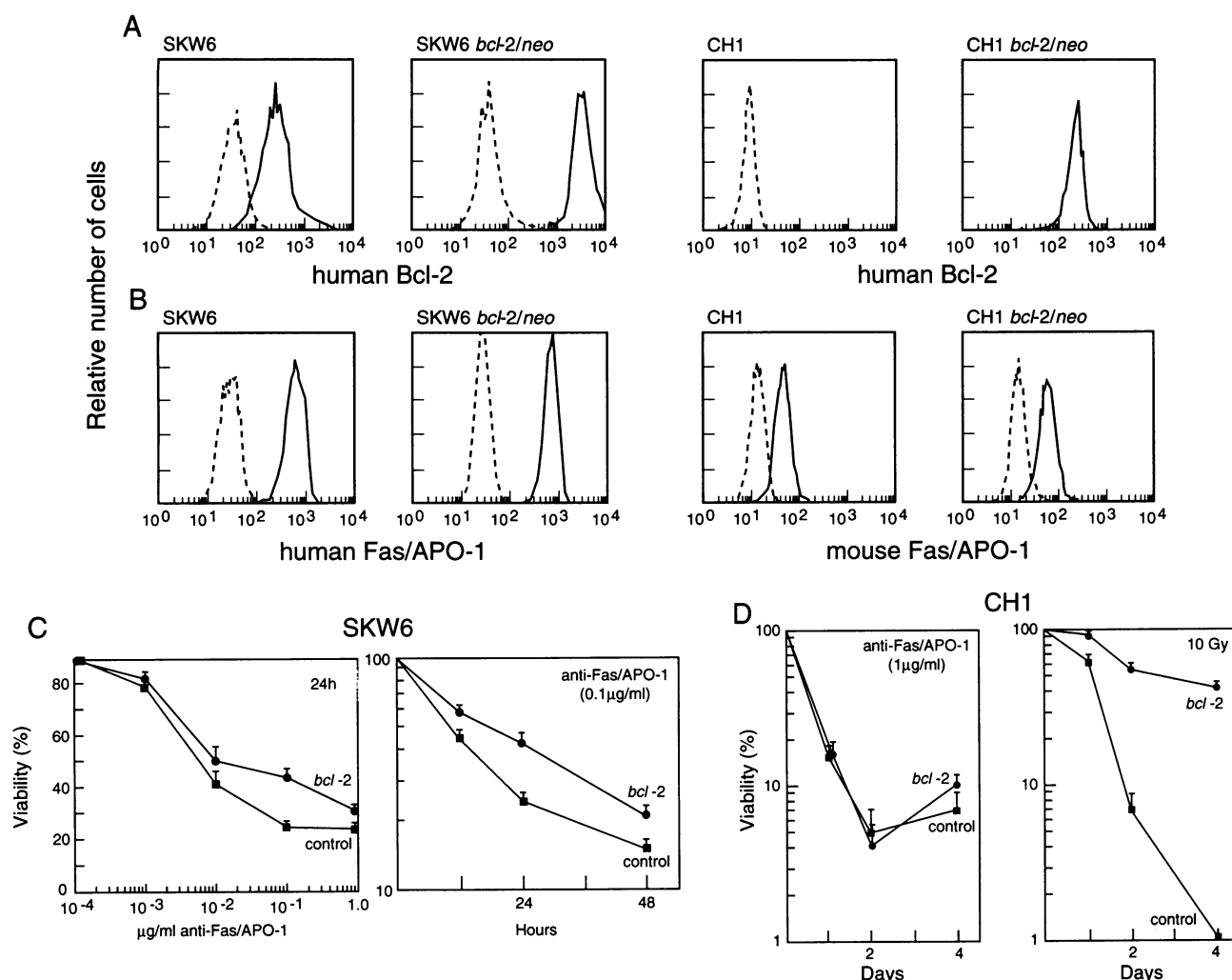
Fig. 1. Bcl-2 does not block Fas/APO-1-transduced killing of B lymphoma cells. Bcl-2 (A) and Fas/APO-1 (B) expression in parental and representative bcl-2 transgene expressing clones of SKW6 and CH1 B lymphoma cells. Human Bcl-2 protein was detected by immunofluorescence staining and flow cytometric analysis of permeabilized cells using Bcl-2–100 mAb. Human and mouse Fas/APO-1 were detected by surface immunofluorescence and flow cytometry, using APO-1 and Jo2 mAbs. The broken lines indicate background staining with an isotype-matched control antibody. Similar levels of Bcl-2 and Fas/APO-1 were detected in several other SKW6 bcl-2/neo and CH1 bcl-2/neo clones. (C) Four independent clones of SKW6 bcl-2/neo cells and as controls, parental SKW6 neo cells and three independent clones of SKW6 neo cells, were treated with 0.1 μg/ml (A) or graded doses (B) of the human Fas/APO-1-specific mAb, APO-1. (D) Three independent lines of CH1 bcl-2/neo cells and, as controls parental CH1 and two independent lines of CH1 neo cells, were treated with 1 μg/ml of Jo2 anti-mouse Fas/APO-1 mAb, or with 10 Gy γ-irradiation. Cell survival was measured after 12, 24 and 48 h (C) or after 1, 2 and 4 days (D) by counting of trypan blue-stained culture aliquots in a haemocytometer.

inhibitor of ICE but not of the cysteine proteases Nedd2/Ich-1 (Miura et al., 1993; Wang et al., 1994) or CPP32 (Nicholson et al., 1995). Mouse CH1 B lymphoma cells were transfected with a CrmA expression construct and several clones expressing CrmA (and parental levels of Fas/APO-1) were selected for further analysis (Figure 4A–C). In contrast to Bcl-2 (Figure 1D), CrmA significantly inhibited Fas/Apo-1 activation-induced apoptosis (Figure 4D), but had no influence on γ-radiation-induced or serum deprivation-induced death (Figure 4E and data not shown). The fact that within one cell line these two apoptosis antagonists reciprocally inhibit cell deaths induced by two stimuli indicates that different proteases are essential for the execution of different cell death stimuli.

Bcl-2 enhances lymphadenopathy in lpr mice
Loss of Fas/APO-1 and gain of Bcl-2 are both cell-autonomous mutations. If Bcl-2 inhibited apoptosis

induced by Fas/APO-1 signalling in lymphocytes, both mutations might be expected to produce a similar phenotype in the whole animal. This is not the case. The marked lymphadenopathy that develops in Fas/APO-1-deficient lpr mice is due largely to the accumulation of non-cycling T cells with an unusual profile of cell surface markers (TCRαβ+, Thy1+, CD4−, CD8−, B220+). Neither lymphadenopathy nor cells of this type could be found at significant levels in bcl-2 transgenic mice (Figure 5A and B).

If Fas/APO-1 and Bcl-2 regulate independent death pathways, one might expect gain of Bcl-2 plus loss of Fas/APO-1 function to be additive or synergistic for lymphocyte accumulation. As illustrated in Figure 5A, lymph node enlargement in lpr mice was substantially increased by a bcl-2 transgene. By the time they were ~3 months old, C3H/HeJ mice carrying the bcl-2 transgene and homozygous for the lpr mutation had 50- to 100-fold
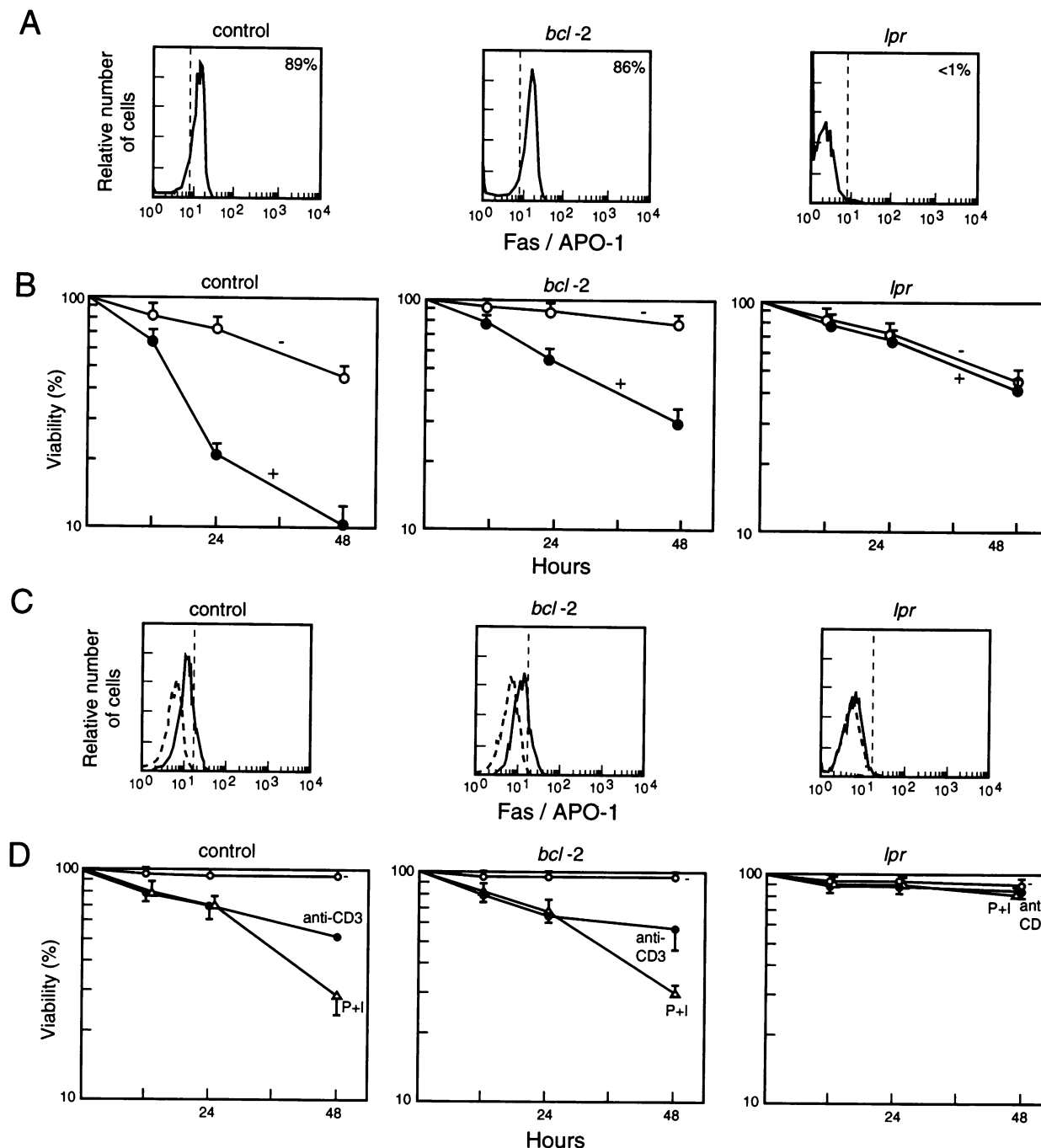
Fig. 2. Bcl-2 does not block Fas/APO-1-transduced killing of thymocytes and activated peripheral T cells. (A) Thymocytes from 5-week-old normal, *bcl-2* transgenic and mutant *lpr* mice (all on the C57BL/6 background) were stained with Jo2 anti-mouse Fas/APO-1 mAb (solid line), or with an isotype matched control antibody (dotted line). (B) Thymocytes from 5-week-old normal, mutant *lpr* and *bcl-2* transgenic mice were incubated in simple tissue culture medium (−) or stimulated with 1 µg/ml anti-mouse Fas/APO-1 mAb (+). Cell survival was determined after 12, 24 and 48 h by propidium iodide staining and flow cytometry. Data represent arithmetic means ± SD from three independent experiments. Similar results were obtained with two independent *bcl-2* transgenic mouse strains. (C) T cells from spleens of 5-week-old normal, *bcl-2-25* transgenic and mutant *lpr* mice (all on the C3H/HeJ background) were activated for 3 days with concanavalin A and then for 2 more days with IL-2. These T lymphoblasts were stained with the anti-mouse Fas/APO-1 mAb (solid line) or with an isotype-matched control antibody (dotted line). (D) Activated T cells (see under C) from normal, *bcl-2-25* transgenic and mutant *lpr* mice were incubated in the presence of IL-2 in wells coated with 20 µg/ml anti-CD3 mAb (+) or with an equal amount of an irrelevant isotype-matched mAb (−) or with PMA plus ionomycin (P+I). Cell survival was determined after 12, 24 and 48 h by propidium iodide staining and flow cytometry. Data represent arithmetic means ± SD from three independent experiments.

more than the normal number of lymph node cells and 5- to 10-fold more than *lpr* littermates lacking the transgene. This remarkable increase was due primarily to further augmentation of the number of unusual T cells (Figure 5B). Thus, while *lpr* lymphocytes are resistant to Fas/APO-1-induced death, they remain vulnerable to physio-

logical signals that induce apoptosis by pathways inhibitable by Bcl-2.

In view of these results, we assessed the sensitivity of *lpr* lymphocytes *in vitro* to a range of cytotoxic agents and asked whether *bcl-2* transgene expression enhanced their resistance. Thymocytes from young *lpr* mice were
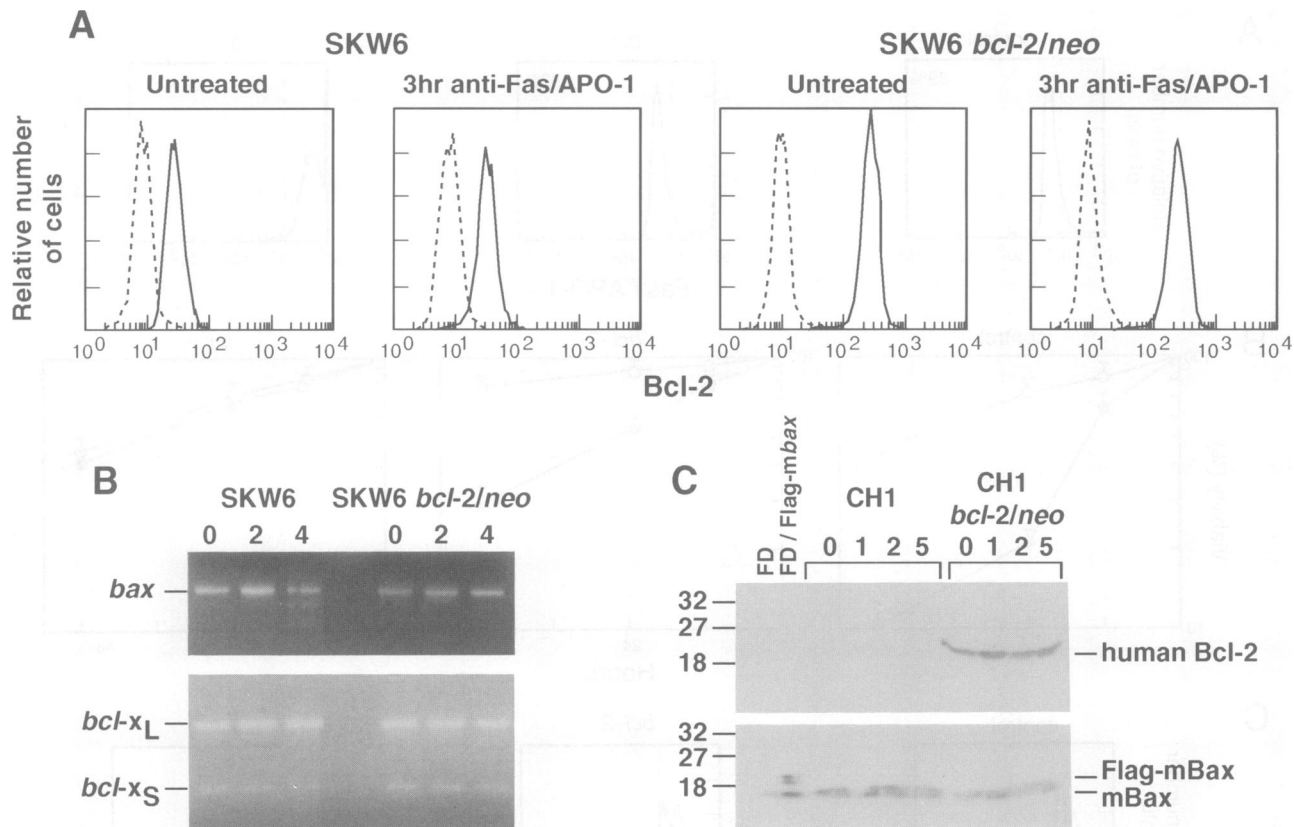
Fig. 3. Fas/APO-1 stimulation does not alter the levels of Bcl-2 or Bax protein, or of *bax*, *bcl-x_S* or *bcl-x_L* mRNA in B lymphoid cell lines.
(**A**) Parental SKW6 and SKW6 *bcl-2/neo* cells were left untreated or treated for 3 h with 1 μg/ml anti-human Fas/APO-1 mAb, APO-1. Bcl-2
protein expression was analysed in permeabilized cells by immunofluorescence staining and flow cytometry using the human Bcl-2 specific mAb,
6C8-28. Similar results were obtained after 1 or 5 h of anti-Fas/APO-1 antibody treatment. (**B**) *Bax*, *bcl-x_L* and *bcl-x_S* mRNA expression was
analysed by RT-PCR in parental SKW6 and SKW6 *bcl-2/neo* cells, which were left untreated, or had been stimulated for 2 or 4 h with 0.1 μg/ml
anti-human Fas/APO-1 antibody. The specific primers used to detect *bax* mRNA and to distinguish *bcl-x_L* from *bcl-x_S* mRNA, and the conditions for
PCR amplification are described in Materials and methods. (**C**) CH1 mouse B lymphoma cells were left untreated or stimulated for 1, 2 or 5 h with
0.1 μg/ml anti-mouse Fas/APO-1 antibody. Lysates from 1×10^6 cells were electrophoresed on a reducing 15% SDS–polyacrylamide gel and proteins
were then transferred by electroblotting on to nitrocellulose membranes. Transgene-encoded human Bcl-2 protein was detected with the mAb
Bcl-2-100, mouse Bax protein with a rabbit antiserum. The specificity of the anti-mouse Bax antiserum was demonstrated with the use of lysates
from the mouse cell line FDC-P1 and from a cloned FDC-P1 subline transfected with a Flag-epitope tagged mouse *bax* expression construct (FDC-
P1 Flag-m*bax*). Parental FDC-P1 cells express the endogenous 18 kDa Bax protein; FDC-P1 Flag-m*bax* cells express the endogenous 18 kDa Bax
protein and the larger 21 kDa transgene-encoded Flag-mBax protein.

as sensitive as normal thymocytes to dexamethasone,
ionomycin, phorbol ester or γ-irradiation (Figure 6A–C,
and data not shown). Moreover, when cultured in the
absence of cytokines, peripheral T cells from *lpr* mice
died with the same kinetics as normal T cells, irrespective
of whether they were TCR$^+$B220$^-$ or TCR$^+$B220$^+$ (Figure
6D and E). In addition, T lymphoblasts generated from
lpr mice died at the same rate as normal T lymphoblasts
when the mitogens were withdrawn (Figure 6F). The *bcl-2*
transgene enhanced the survival of all these types of *lpr*
T cells, but their resistance was no greater than that of
otherwise normal T cells expressing the *bcl-2* transgene
(Figure 6). These results establish that Fas/APO-1 is not
involved in apoptosis induced in T cells by dexamethasone,
ionomycin, ionizing radiation, PMA or cytokine with-
drawal, and reiterate the apparent independence of the
Fas/APO-1 death pathway from that inhibitable by Bcl-2.

Autoreactive thymocytes are deleted in lpr mice expressing a bcl-2 transgene

The *lpr* mutation does not inhibit negative selection of
thymocytes, i.e. the deletion of autoreactive cells (Sidman

et al., 1992; Herron *et al.*, 1993); and neither does *bcl-2*
transgene expression block this process, despite delaying
it (Sentman *et al.*, 1991; Strasser *et al.*, 1991, 1994). If
Fas/APO-1 and Bcl-2 regulate independent pathways, as
our results suggested, it seemed possible that the combined
effect of gain of Bcl-2 and loss of Fas/APO-1 function
might block the deletion of self-reactive T cells. Accord-
ingly, we investigated the fate of Mls self antigen-specific
thymocytes in C3H/HeJ-*lpr* mice expressing the *bcl-2*
transgene. T cells that display a Vβ3-bearing receptor
specifically recognize the minor lymphocyte stimulatory
antigen Mls-2 associated with I–E class II major histo-
compatibility complex molecules (Pullen *et al.*, 1988).
C3H/HeJ mice express Mls-2 and I-E^k, and delete Vβ3-
bearing T cells to a level below 0.1% of all T cells in
spleen and lymph nodes. In contrast, C57BL/6 mice,
which lack Mls-2 and I-E, contain sizeable numbers of
such cells (3–5% of all T cells) in peripheral lymphoid
organs (data not shown, and Pullen *et al.*, 1988). Sig-
nificantly, Vβ3-bearing T cells could not be detected in
the lymph nodes and spleen of C3H/HeJ-*lpr* mice harbour-
ing the *bcl-2* transgene (<0.1% of all T cells, data not
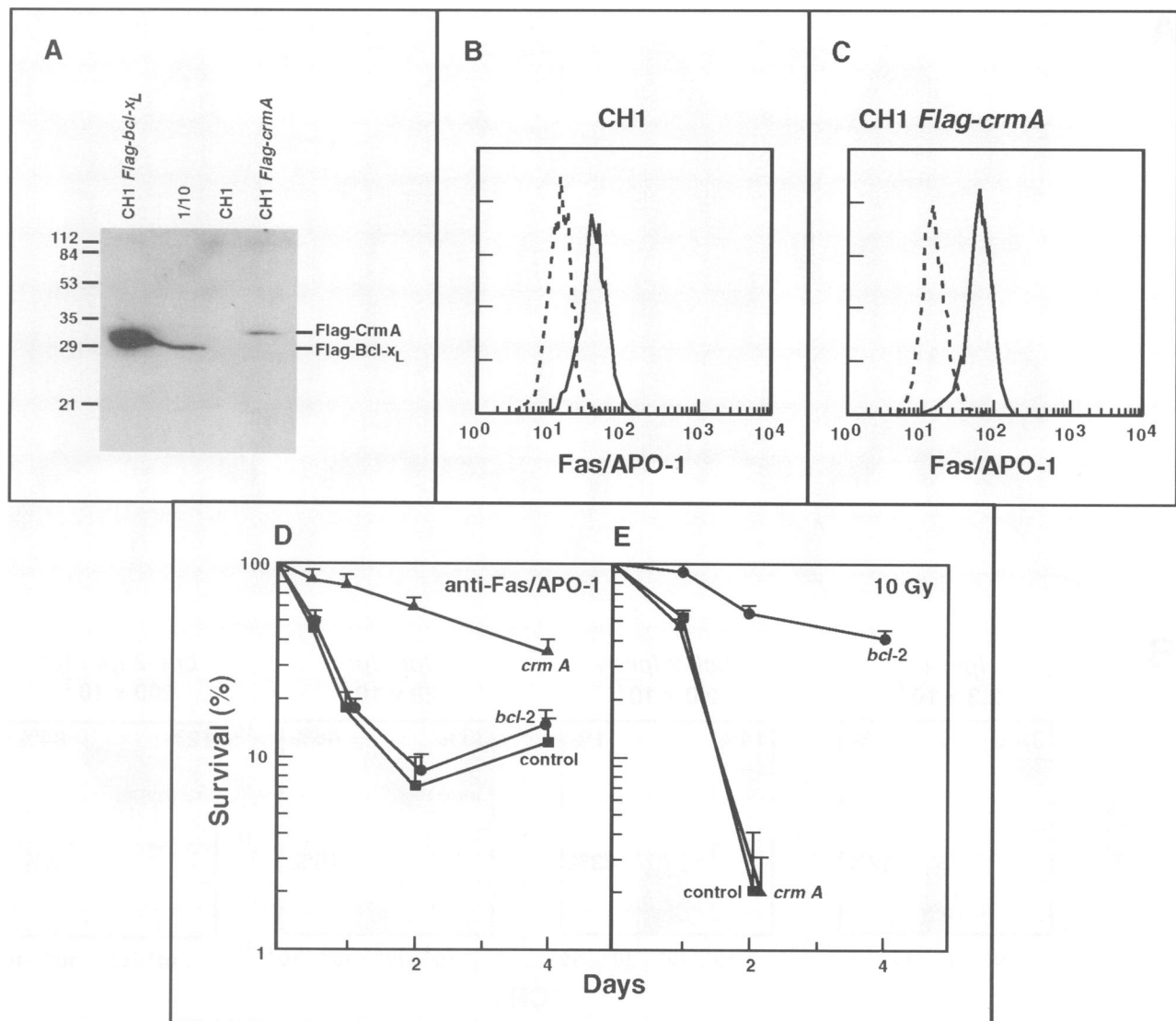
Fig. 4. *CrmA* transgene expression inhibits Fas/APO-1-induced but not γ-radiation-induced apoptosis of CH1 B lymphoma cells. CrmA (A) and Fas/APO-1 (B and C) expression in parental CH1 cells and a representative CH1 *Flag-CrmA/puro* line. (A) Flag-CrmA protein was detected by Western blotting with a Flag-specific mAb. CH1 *Flag-bcl-x_L/puro* cells were used as a positive control. Lysates from 1×10^6 or 1×10^5 (1/10) cells were loaded per lane. (B and C) Mouse Fas/APO-1 expression was detected in parental CH1 (B) and in a representative CH1 *Flag-CrmA/puro* line (C) by surface immunofluorescence and flow cytometry, using mAb Jo2 (solid line). Background staining with an isotype-matched control mAb is shown in broken lines. (D and E) Three independent lines of CH1 *Flag-crmA/puro* cells, three independent lines of CH1 *bcl-2/neo* cells and as controls, parental CH1 cells and two independent lines of CH1 *neo* cells, were treated with 1 μg/ml of the mouse Fas/APO-1-specific mAb, Jo2 (D), or with 10 Gy γ-irradiation (E). Cell viability was measured after 12 h, 1, 2 and 4 days by counting of trypan blue-stained cell culture aliquots in a haemocytometer.

shown). Thus, the combination of Fas/APO-1 deficiency and Bcl-2 excess does not abrogate negative selection in the thymus.

Loss of Fas/APO-1 and gain of Bcl-2 synergize to promote survival of chronically activated T lymphocytes

Upon activation with superantigens such as staphylococcal enterotoxin B (SEB), specific T cells initially multiply, but subsequently most undergo apoptosis; hence their number drops below that before immunization (Kawabe and Ochi, 1991). Survival of activated peripheral T cells is regulated both positively (via cytokine receptors) and negatively (via Fas/APO-1). Although Bcl-2 inhibits apoptosis induced by cytokine deprivation in T lympho-

blasts *in vitro*, T cell responses to SEB are only moderately prolonged in *bcl-2* transgenic mice (Strasser *et al.*, 1991). Similarly, deletion of SEB-activated peripheral T cells is impaired but not abrogated in *lpr* mice (Scott *et al.*, 1993). To test whether loss of Fas/APO-1 and gain of Bcl-2 co-operate to promote the survival of chronically activated T cells, we examined the peripheral T cell response to SEB in *bcl-2/lpr* mice and their littermates. Mice were injected three times with either SEB or saline, and then TCRVβ8-expressing CD4⁺CD8⁻ T cells, which dominate the SEB response (Kawabe and Ochi, 1991), were enumerated after 7 days (Table I). In normal mice, CD4⁺CD8⁻Vβ8⁺ T cell numbers dropped by 65% after injection of SEB. A smaller reduction was apparent in SEB-injected *lpr* mice (30%) and in *bcl-2* transgenic mice

A

B
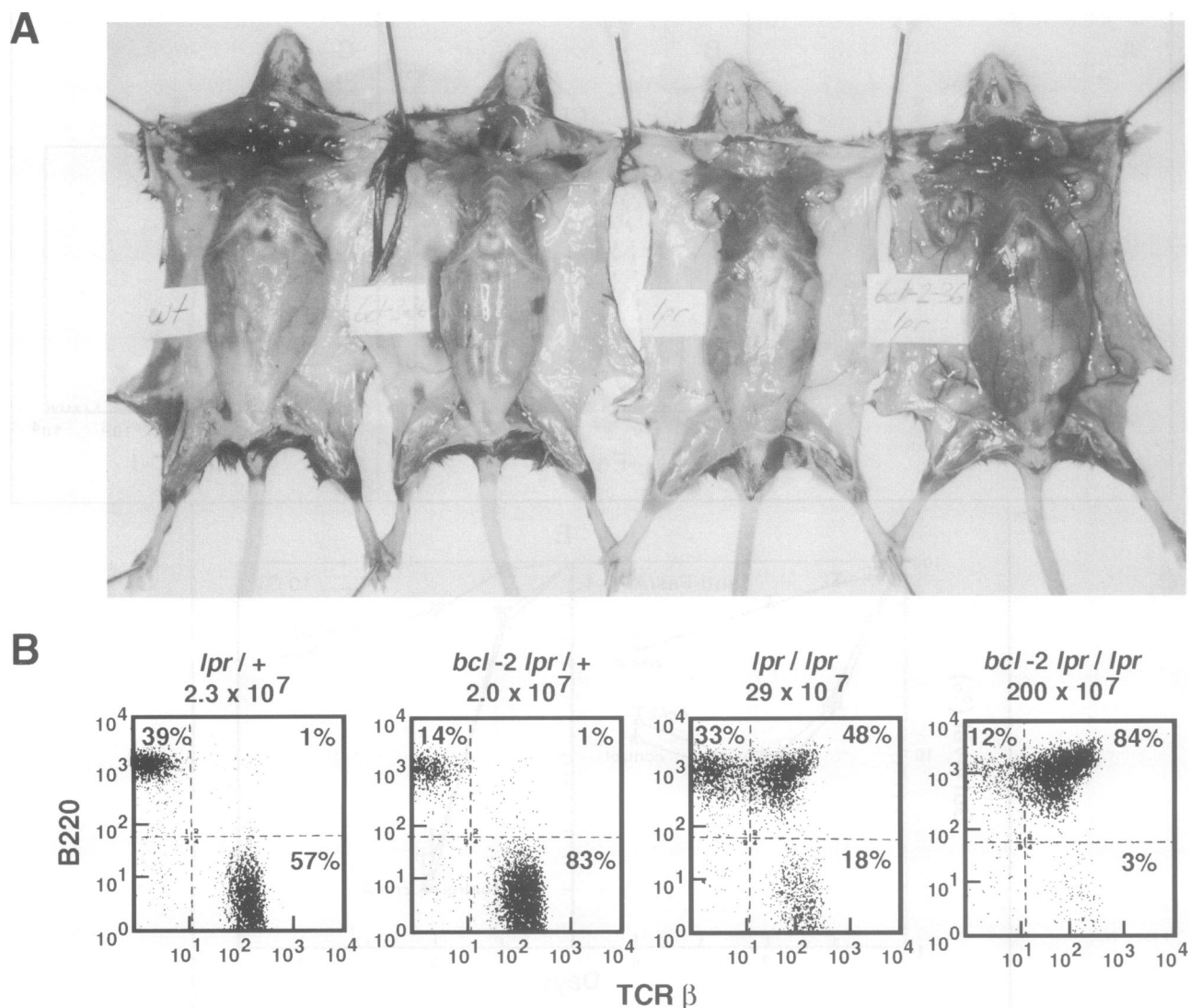
Fig. 5. *Bcl-2* transgene expression enhances lymphadenopathy in *lpr* mice. (**A**) Necropsy of a control, a *bcl-2*, a mutant *lpr* and a *bcl-2/lpr* mouse (all 97 days old and on the C3H/HeJ background, N6). The spleen weights were 100, 150, 350 and 1080 mg, respectively. These mice were representative of >10 animals of each genotype that were examined. Similar results were obtained with two independent *bcl-2* transgenic strains. (**B**) Immunofluorescence staining and flow cytometric analysis of lymph node cells from a control, a *bcl-2*, a mutant *lpr* and a *bcl-2/lpr* mouse (all 97 days old and on the C3H/HeJ background). The total number of leukocytes in the collection of inguinal, axillary, brachial and mesenteric lymph nodes of all animals is indicated above each FACS profile. Lymph node cells were stained with a PE-labelled mAb specific to CD45R-B220 (RA3-6B2) and a FITC-labelled mAb specific to the TCRβ chain (H57-59721).

(40%). In contrast, in *bcl-2* transgenic *lpr* mice, the total number of splenic TCRVβ8$^+$ CD4$^+$CD8$^-$ T cells was not reduced but increased 3- to 4-fold after injection of SEB (Table I). This increase was antigen-specific, since the number of TCRVβ6$^+$ CD4$^+$CD8$^-$ T cells, which do not respond to SEB (Kawabe and Ochi, 1991), was unchanged (Table I). These data demonstrate that Bcl-2 and Fas/APO-1 regulate independent pathways determining the survival of antigen-activated peripheral T cells and provide evidence that the abnormal accumulation of T cells in *lpr* and especially *bcl-2/lpr* mice results from defective physiological killing of activated peripheral T cells.

Discussion

Bcl-2 is a poor inhibitor of the death signal transduced by Fas/APO-1

The death process induced by activation of Fas/APO-1 displays the classic features of apoptosis: chromatin com-

paction, condensation of the cytoplasm, membrane ruffling and DNA fragmentation (Trauth *et al.*, 1989; Yonehara *et al.*, 1989). Since Bcl-2 can inhibit apoptosis induced by diverse cytotoxic stimuli, including γ-irradiation, glucocorticoids and cytokine deprivation (Vaux *et al.*, 1988; Sentman *et al.*, 1991; Strasser *et al.*, 1991 and Figures 1 and 6), it has been argued that there is a final effector mechanism for apoptosis and that Bcl-2 acts within this common pathway. Surprisingly, however, lymphoid cells overexpressing Bcl-2 remained vulnerable to the action of Fas/APO-1. This was demonstrated not only for two B lymphoma cell lines, but also for normal thymocytes and mitogen-activated peripheral T cells (Figures 1 and 2). Susceptibility did not appear to be due to inhibition of Bcl-2, because levels of Bcl-2 remained high in cells treated with Fas/APO-1-specific antibodies and there was no increase in the expression of Bax or Bcl-x$_S$, two dominant repressors of Bcl-2 (Figure 3).
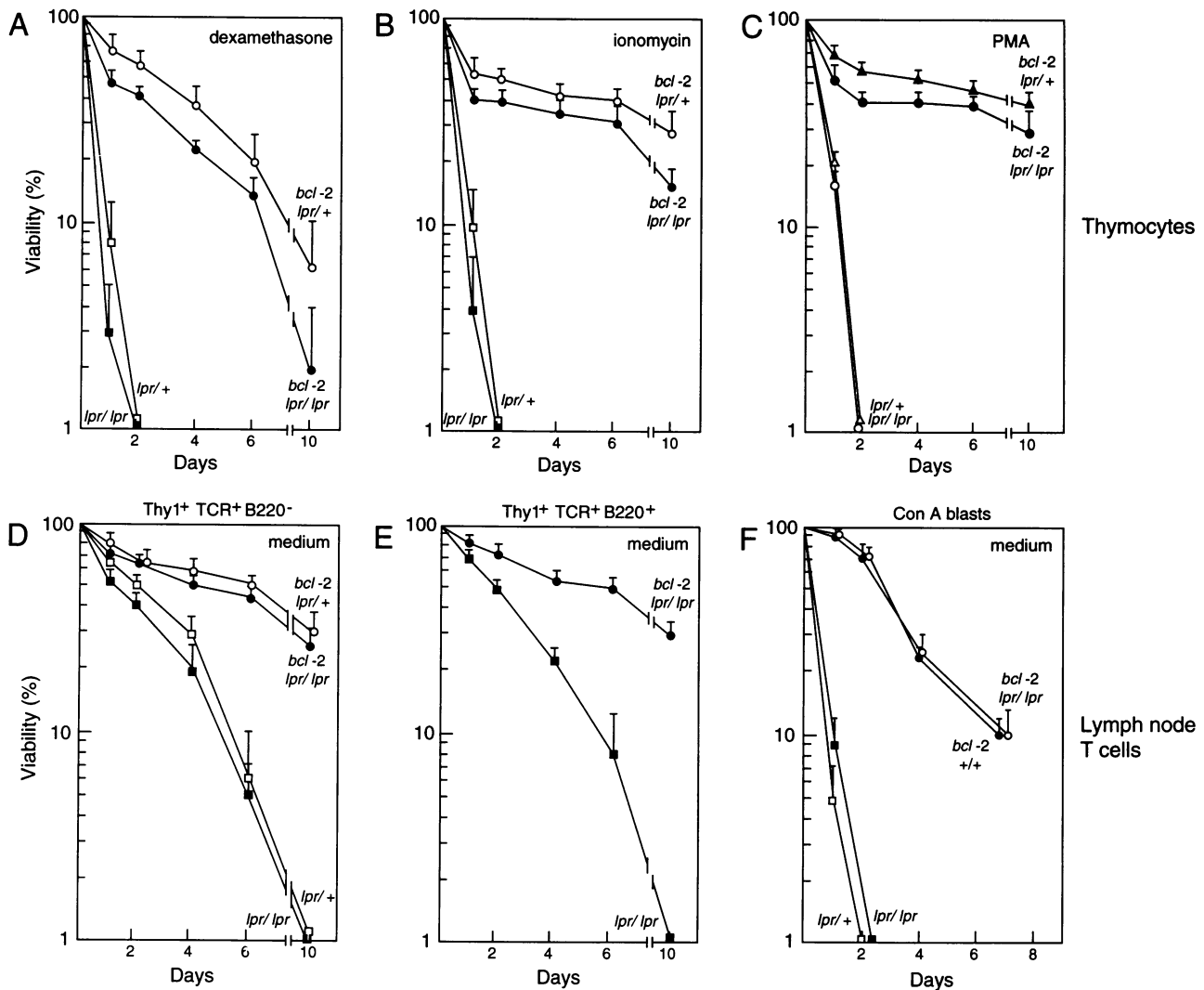
Fig. 6. Loss of Fas/APO-1 does not enhance the resistance of *bcl-2* transgenic lymphocytes to growth factor deprivation or cytotoxic stimuli. (A–C) Thymocytes from *lpr/+*, *bcl-2/lpr/+*, *lpr/lpr* and *bcl-2/lpr/lpr* mice (all on the C3H/HeJ background, N2) were treated in simple tissue culture medium with 1 μM dexamethasone (A), 1 μg/ml ionomycin (B) or 2 ng/ml PMA (C). (D) Conventional Thy1$^+$B220$^-$ T cells were FACS-sorted from lymph nodes of young (<35 days) *lpr/+*, *bcl-2/lpr/+*, *lpr/lpr* and *bcl-2/lpr/lpr* mice, and cultured in simple tissue culture medium. (E) TCRαβ$^+$B220$^+$ T cells were FACS-sorted from lymph nodes of older (>80 days) *lpr/lpr* and *bcl-2/lpr/lpr* mice, and cultured in simple tissue culture medium. (F) T lymphoblasts were prepared from young (<35 days) *lpr/+*, *bcl-2/lpr/+*, *lpr/lpr* and *bcl-2/lpr/lpr* mice by stimulation for 3 days with concanavalin A and for a further 2 days with IL-2. These cells were then cultured in simple medium after growth factors and mitogens had been removed. Cell survival was determined on days 1, 2, 4 and 7 by trypan blue exclusion and counting in a haemocytometer. Data represent arithmetic means ± SD from cells of three animals of each genotype. Similar results were obtained with two independent *bcl-2* transgenic strains.

Bcl-2 has also been observed to be a poor inhibitor of the death signal transduced by TNF (Vanhaesebroeck *et al.*, 1993 and our unpublished observations). Fas/APO-1 and TNF-RI (but none of the other known members of this receptor family) have a conserved cytoplasmic region of ~70 amino acid residues, dubbed the 'death domain', that is essential for transmitting a death signal (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). The death domain apparently mediates association of the receptors with cytoplasmic effectors that contain similar motifs: Mort1/FADD (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995) and TRADD (Hsu *et al.*, 1995), which uniquely associate with Fas/APO-1 and TNF-R1 respectively, and RIP (Stanger *et al.*, 1995) which can bind to either. Overexpression of these proteins promotes apoptosis and, consistent with our findings and those of Vanhaesebroeck *et al.* (1993), apoptosis induced by either TRADD- or Mort1/FADD can

be blocked by CrmA (Chinnaiyan *et al.*, 1995; Hsu *et al.*, 1995), a potent inhibitor of the cysteine protease ICE (see below), but not by Bcl-2, at least in the case of TRADD (Hsu *et al.*, 1995).

Others have reported that death induced in lymphoid cells via Fas/APO-1 can be partially blocked by Bcl-2 (Itoh *et al.*, 1993). Furthermore, substantial inhibition of killing by either Fas/APO-1 or TNF has been seen in non-lymphoid cells (Jäättelä *et al.*, 1995; Vandenabeele *et al.*, 1995). The apparent discrepancy with the results we report here may reflect differences in cell death circuitry between cell lines. More specifically, these receptors may be able to trigger two pathways to apoptosis, only one of which is Bcl-2-sensitive, and the relative contributions of the pathways may vary between cell lines. Ceramide, a product of sphingomyelin hydrolysis, has been implicated as a second messenger in both the Fas/APO-1 and TNF-R1

Table I. Loss and persistence of antigen-reactive T cells after immunization

Genotype	Treatment CD4$^+$8$^-$Vβ8$^+$ cells ($\times 10^{-6}$)		Treatment CD4$^+$8$^-$Vβ6$^+$ cells ($\times 10^{-6}$)	
	Saline	SEB	Saline	SEB
Wild-type	5.6 ± 2.0	2.0 ± 0.3	2.6 ± 0.6	1.9 ± 0.2
bcl-2	5.8 ± 1.4	3.5 ± 0.2	2.2 ± 0.5	1.8 ± 0.2
lpr	7.2 ± 3.3	5.0 ± 2.0	1.6 ± 0.3	1.5 ± 0.4
bcl-2/lpr	6.8 ± 1.4	23 ± 2	2.4 ± 0.4	2.3 ± 0.3

Three mice of each genotype: wild-type, *bcl-2* transgenic, *lpr* and *bcl-2* transgenic *lpr* (5–7 weeks old), were injected i.p. on days 0, 1 and 2 with saline or with 10 μg staphylococcal enterotoxin B (SEB). On day 7, total numbers of splenic CD4$^+$8$^-$TCRVβ8$^+$ and CD4$^+$8$^-$TCRVβ6$^+$ T cells were determined by haemocytometer counting of total cells and flow cytometry. Numbers are arithmetic means ± SD. Similar results were obtained with two independent *bcl-2* transgenic strains.

pathways (Obeid *et al.*, 1993; Jarvis *et al.*, 1994; Gulbins *et al.*, 1995) and, intriguingly, ceramide-induced apoptosis of CH1 B lymphoma cells is unaffected by CrmA but can be inhibited by Bcl-2 (our unpublished results).

Gain of Bcl-2 function compounds the effects of Fas/Apo-1 deficiency

Enhanced lymphocyte survival resulting from loss of Fas/APO-1 function evokes a singular phenotype in the whole animal: a progressive lymphadenopathy due primarily to the accumulation of B220$^+$ T cells. When *lpr* mice were crossed with *bcl-2* transgenic mice, offspring carrying both mutations developed an astonishing number of these cells. By the time they were 3 months old, they had ~100-fold more peripheral T cells than normal mice and 10 times more than their *lpr* littermates (Figure 5B). We observed that gain of Bcl-2 and loss of Fas/APO-1 synergized to promote survival of chronically stimulated T cells *in vivo* (Table I), which suggests that the massive accumulation of lymphocytes probably results from a failure to eliminate T cells after their activation in immune responses. Resting T cells from *bcl-2/lpr* mice were as resistant as those from *bcl-2* transgenic mice to cytokine deprivation or exposure to cytotoxic stimuli (Figure 6), while those from *lpr* mice died rapidly under these conditions. Thus, *lpr* T cells remain sensitive to many apoptosis-inducing stimuli. The accumulation of T cells in *lpr* mice must be limited by other mechanisms that normally act in addition to Fas/APO-1 to eliminate T cells at the end of an immune response.

Roles of Bcl-2 and Fas/APO-1 in lymphocyte development and function

Both Bcl-2 and Fas/APO-1 regulate lymphocyte survival. Bcl-2 can counter 'death by neglect', since expression of a *bcl-2* transgene preserves immature CD4$^+$8$^+$ thymocytes in a non-selecting MHC background (Linette *et al.*, 1994; Strasser *et al.*, 1994), but Bcl-2 does not block apoptosis of self-reactive T cells because no such cells emerge from the thymus in *bcl-2* transgenic mice (Sentman *et al.*, 1991; Strasser *et al.*, 1991, 1994). Fas/APO-1 is expressed by most thymocytes (Ogasawara *et al.*, 1993). Since Fas/APO-1-induced apoptosis is insensitive to inhibition by Bcl-2, could activation of Fas/APO-1 be the mechanism

for inducing death in self-reactive thymocytes? Seemingly not, since *lpr* mice lack self-reactive T cells (Sidman *et al.*, 1992; Herron *et al.*, 1993; Singer and Abbas, 1994), even when they express a *bcl-2* transgene (as shown here). Deletion of autoreactive thymocytes also occurs normally in mutant mice lacking the p55 or the p75 TNF receptor (Pfeffer *et al.*, 1993; Rothe *et al.*, 1993; Erickson *et al.*, 1994). Killing of autoreactive thymocytes may therefore be triggered via independent activation of several members of the TNF receptor family or through a yet unknown mechanism.

Physiological cell death also helps to terminate immune responses in peripheral lymphoid organs. One mechanism for killing activated T cells is deprivation of survival-promoting cytokines. This form of cell death can be inhibited by Bcl-2 but not by loss of Fas/APO-1 (Figure 6F). In contrast, the other mechanism for killing T lymphoblasts—apoptosis induced by antigen receptor activation—depends on Fas/APO-1 stimulation (Russell and Wang, 1993; Russell *et al.*, 1993; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995), but is unaffected by Bcl-2 (Figure 2D). The existence of these two independently regulated killing mechanisms can explain why T cell responses to superantigens are only moderately prolonged in *bcl-2* transgenic animals (Strasser *et al.*, 1991) and *lpr* mice (Scott *et al.*, 1993), and why loss of Fas/APO-1 and gain of Bcl-2 synergize to promote the survival of chronically activated T cells (Table I). In normal mice, T cells may lose CD4 and CD8 co-receptors and express B220 some time after activation, but be killed so rapidly by Fas/APO-1 ligand that they have hitherto escaped detection.

Implications for the molecular mechanisms of cell death regulation

It now seems clear that apoptosis in vertebrates involves a family of cysteine proteases homologous to that encoded by *ced-3*, one of two genes essential for cell death in *C.elegans* (Hengartner and Horvitz, 1994b). As well as the prototype, ICE (Yuan *et al.*, 1993), the growing family includes Nedd2/Ich-1, CPP32, Tx/ICE-$_{rel}$II, ICE-$_{rel}$III and Mch2α (Fernandes-Alnemri *et al.*, 1994, 1995; Kumar *et al.*, 1994; Wang *et al.*, 1994; Faucheu *et al.*, 1995; Munday *et al.*, 1995), each of which can provoke apoptosis when expressed at high levels in mammalian cells. Several of the proteases can cleave both their own and others' precursors (Thornberry *et al.*, 1992; Faucheu *et al.*, 1995; Gu *et al.*, 1995), raising the possibility that apoptosis is initiated by an ordered cascade of precursor activation and subsequently sustained by autocatalysis. The network may vary in different cell types, since Nedd2/Ich-1 did not induce apoptosis in all cell lines tested (Kumar *et al.*, 1994; Wang *et al.*, 1994). Very little is known about the substrates of ICE-like proteases, particularly the target(s) relevant to apoptosis.

How Bcl-2 and Bcl-x$_L$ block apoptosis is unknown, but they might interfere with the function of the proteases, their activators or their substrates (Cory *et al.*, 1994). In view of the ability of Bcl-2 to block apoptosis induced by so many other signals, why does it fail to block apoptosis triggered by Fas/Apo-1 and TNF-R1 (Vanhaesebroeck *et al.*, 1993; Hsu *et al.*, 1995; this paper)? To date we have found no indication that Fas/Apo-1

ligation induces a Bcl-2 repressor such as Bax. Rather, our data suggest that Fas/APO-1 signalling simply bypasses the Bcl-2 checkpoint.

There is increasing evidence that apoptosis triggered via Fas/Apo-1 or TNF-RI depends on ICE or a close homologue of ICE. The initial data supporting this idea came from studies utilizing CrmA, the viral serpin-like inhibitor of ICE (Ray et al., 1992), recently shown to have a 10 000-fold preference for ICE over CPP32 (Nicholson et al., 1995). CrmA enhanced the survival of breast carcinoma cells and several lymphoid lines treated with anti-Fas/APO-1 antibody or TNF (Enari et al., 1995; Los et al., 1995; Tewari and Dixit, 1995) and, as mentioned above, blocked apoptosis induced by overexpression of TRADD or Mort1/FADD (Chinnaiyan et al., 1995; Hsu et al., 1995). CrmA also inhibited induction of apoptosis in chicken dorsal root ganglion cells by growth factor deprivation (Gagliardini et al., 1994). ICE does not, however, play an essential role in apoptosis induced by all stimuli since, although thymocytes from ICE-deficient mice are resistant to Fas/APO-1-transduced apoptosis, they remain sensitive to apoptosis induced by corticosteroids or γ-irradiation (Kuida et al., 1995; Li et al., 1995). ICE is also dispensable for programmed cell deaths during normal development (Kuida et al., 1995; Li et al., 1995) and CPP32 rather than ICE appears to be necessary for apoptosis induced by camptothecin in osteosarcoma cells (Nicholson et al., 1995).

If there is but a single biochemical pathway for apoptosis, these considerations suggest that ICE (and/or its close relatives) participate at a point downstream from the step inhibitable by Bcl-2. Alternatively, ICE may participate within a biochemically distinct pathway lacking the reaction inhibitable by Bcl-2. The development of specific inhibitors of the other cell death proteases should make it feasible to distinguish between these possibilities.

Materials and methods

Mice

The derivation and characteristics of the Eμ-bcl-2-25 and Eμ-bcl-2-36 transgenic mice, which express high levels of human Bcl-2 constitutively in T cells, and B plus T cells, respectively (Strasser et al., 1991), and the mutant lpr mice on C3H/HeJ and C57BL/6J backgrounds (Cohen and Eisenberg, 1993) have previously been described. The bcl-2/lpr mice were generated by serially crossing bcl-2 mice with C3H/HeJ-lpr mice. Inheritors of the bcl-2 transgene were identified by PCR on blood leukocyte DNA using oligonucleotide primers for the SV40 sequence included in the transgene. Mice homozygous for the lpr mutation were identified by PCR amplification of blood leukocyte DNA as described by Singer and Abbas (1994).

Cell lines and transfections

Cell lines were cultured in the high glucose version of Dulbecco's modified Eagle's (DME) medium supplemented with 13 μM folic acid, 250 μM L-asparagine, 50 μM 2-mercaptoethanol and 10% fetal bovine serum (FBS). The human B lymphoblastoid cell line SKW6 (Trauth et al., 1989) was transfected by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA) with an expression construct containing the human bcl-2 cDNA and neo, or with a control neo construct (15 μg DNA/5×10⁶ cells in 0.5 ml buffered saline). Independent clones of transfected cells were selected with G418 (1.5 mg/ml; Sigma, St Louis, MO) and limit dilution plating. Mouse CH1 B lymphoma cells were infected with a retrovirus expressing the human bcl-2 cDNA and neo or with a control neo retrovirus by incubating cells (1×10⁶/ml) with supernatant from retrovirus-producing fibroblasts (titres 2×10⁵/ml and 1×10⁶/ml, respectively) containing 4 μg/ml polybrene (Sigma). Separate lots of CH1 cells were transfected with an expression vector

(pEFPGKpuro) derived from pEFBos (Mizushima and Nagata, 1990) and containing crmA cDNA (Ray et al., 1992) tagged with the Flag epitope (Blanar and Rutter, 1992), or with a control puro construct. After 48 h, independent polyclonal lines of infected cells were selected by addition of either G418 (1.5 mg/ml) or puromycin (2 μg/ml).

Cell survival and proliferation assays

Cell proliferation was measured as [³H]thymidine incorporation after incubating 100 μl cultures (starting concentration 1×10⁵ cells/ml) in 96-well microtitre plates for 6 h with 0.5 μCi [³H]thymidine (Amersham, UK). Cell viability was determined either by trypan blue exclusion and counting in a haemocytometer or by flow cytometric analysis of propidium iodide-stained cells using a FACScan (Becton Dickinson, Mountain View, CA). Lymphocytotoxicity was tested in vitro with anti-human Fas/APO-1 mAb APO-1 (Trauth et al., 1989) and anti-mouse Fas/APO-1 mAb Jo2 [Pharmingen, San Diego, CA; (Ogasawara et al., 1993)] within the concentration range of 0.0001–10 μg/ml, phorbol 12-myristate 13-acetate (Sigma) at 2 ng/ml, ionomycin (Sigma) at 1 μg/ml, dexamethasone (Sigma) at 1 μM, or γ-irradiation with 10 Gy from a ⁶⁰Co source at a rate of 3 Gy/min. T lymphocytes were activated in vitro for 3 days with 2 μg/ml concanavalin A (Pharmacia, Uppsala, Sweden) and then for an additional 2 days in 100 U/ml recombinant mouse IL-2. Growth factor deprivation-induced apoptosis was studied in such T lymphoblasts after removing IL-2 by repeated (3×) centrifugation through FBS. Antigen receptor activation-induced apoptosis was triggered by culturing T lymphoblasts on plates coated with 20 μg/ml anti-CD3 mAb (KT3) or by stimulation with PMA (2 ng/ml) plus ionomycin (1 μg/ml).

Immunofluorescence staining and flow cytometric analysis

Dispersed cells from thymus, bone marrow, spleen and lymph nodes were surface-stained as previously described (Strasser et al., 1991), either with directly fluoresceinated mAbs (5.1 anti-μ; 53-6.7 anti-CD8; 30H12 anti-Thy1.2; H57.597.2.1 anti-TCRβ-chain) or with biotinylated mAbs (RA3-6B2 anti-CD45R-B220, GK-1.5 anti-CD4; KJ25 anti-TCRVβ3, F23.1 anti-TCRVβ8, F23.2 anti-TCRVβ8.2) followed by R-phycoerythrin–streptavidin (Caltag, San Francisco, CA). Between 5000–20 000 viable cells (not stained by propidium iodide) were analysed in the FACScan flow cytometer. Subsets of cells were purified by flow cytometric sorting using a FACS II or a FACStar plus (Becton Dickinson). For cytoplasmic immunofluorescence detection of the human and mouse Bcl-2 and Bax proteins, cells were fixed either for 5 min in 80% methanol at −20°C or for 10 min in 1% paraformaldehyde at room temperature and then permeabilized with 0.3% saponin (Sigma), which was included in all subsequent staining and washing steps. The cells were first stained with the primary mAbs Bcl-2-100 [mouse anti-human Bcl-2 (Pezzella et al., 1990); 6C8-28 (hamster anti-human Bcl-2, Pharmingen; Oltvai et al., 1993); or 3F11 (hamster anti-mouse Bcl-2, Pharmingen; Oltvai et al., 1993)] at a concentration of 2–5 μg/ml for 40 min on ice and then with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1–2 μg/ml; Southern Biotechnology, Birmingham, AL) or FITC-conjugated mouse anti-hamster IgG (1–2 μg/ml; Pharmingen) antibodies as secondary reagents. Mouse and human Bax proteins were detected with two rabbit antisera and FITC-conjugated sheep anti-rabbit IgG (1–2 μg/ml; Silenus). Cells were analysed in the FACScan, live and dead cells being discriminated on the basis of their forward and side light-scattering properties. Apoptotic cells were quantified by flow cytometry. Cells were stained for 30 min at 4°C with 50 μg/ml propidium iodide (Sigma) in 0.1% sodium acetate with 0.2% Triton X-100 (BDH Chemicals, UK) and stained cells were analysed in the FACScan.

Western blotting

Proteins were solubilized from control and anti-Fas/APO-1 stimulated cells by lysis in gel-running buffer (0.25 M Tris–HCl, pH 6.8, 1% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue), size fractionated on 12.5 or 15% SDS–polyacrylamide gels and then transferred to nitrocellulose membranes by electroblotting. After blocking (>1 h at room temperature in 5% skim milk, 1% FBS, 1% casein), membranes were incubated (>2 h at room temperature) with antibodies (2–10 μg/ml) specific for human or mouse Bcl-2 (see above for clones) or with rabbit antisera (dilution 1/100 to 1/500) specific for human or mouse Bax (see above). Flag-tagged proteins were detected with anti-Flag M2 mAb (Eastman Kodak, New Haven, CT). When mouse and hamster mAbs were used as primary reagents, membranes were subsequently incubated (>1 h at room temperature) with 5–10 μg/ml affinity-purified rabbit anti-mouse IgG (Fcγ-specific) or rabbit anti-

hamster IgG antibodies (both from Jackson ImmunoResearch). Membranes were finally probed (>1 h at room temperature) with ^{125}I-labelled protein A (1–2×10^6 c.p.m./ml).

RNA extraction and PCR analysis

Total RNA was prepared by guanidinium isothiocyanate extraction from parental SKW6 and SKW6 bcl-2/neo cells that were left untreated or had been stimulated for 2, 4 or 7 h with 0.1 µg/ml anti-Fas/APO-1 antibody. RT–PCR analysis was performed to detect *bax* (primers: 5′-GGA GAA TTC GTG ATG GAC GGG TCC GGG GAG CAG CCC AGA GGC-3′ and 5′- GCA GCG TCG ACC TCA GCC CAT CTT CTT CCA GAT GGT GAG CGA-3′) or *bcl-x$_L$* and *bcl-x$_S$* (primers: 5′-TGA GGG AGG CAG GCG ACG AGT TTG A-3′ and 5′-CCA CAG TCA TGC CCG TCA GGA ACC A-3′) mRNA.

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References

Blanar,M. and Rutter,W. (1992) *Science*, **256**, 1014–1018.

Boise,L.H., Gonzalez-Garcia,M., Postema,C.E., Ding,L., Lindsten,T., Turka,L.A., Mao,X., Nuñez,G. and Thompson,C.B. (1993) *Cell*, **74**, 597–608.

Boldin,M.P., Varfolomeev,E.E., Pancer,Z., Mett,I.L., Camonis,J.H. and Wallach,D. (1995) *J. Biol. Chem.*, **270**, 7795–7798.

Brunner,T. *et al.* (1995) *Nature*, **373**, 441–444.

Chinnaiyan,A.M., O'Rourke,K., Tewari,M. and Dixit,V.M. (1995) *Cell*, **81**, 505–512.

Chittenden,T., Harrington,E.A., O'Connor,R., Flemington,C., Lutz,R.J., Evan,G.I. and Guild,B.C. (1995) *Nature*, **374**, 733–736.

Cohen,P.L. and Eisenberg,R.A. (1993) *Immunol. Today*, **13**, 427–428.

Cory,S., Strasser,A., Jacks,T., Corcoran,L.M., Metz,T., Harris,A.W. and Adams,J.M. (1994) *Cold Spring Harbor Symp. Quant. Biol.*, **59**, 365–375.

Dhein,J., Walczak,H., Bäumler,C., Debatin,K.-M. and Krammer,P.H. (1995) *Nature*, **373**, 438–441.

Ellis,R.E., Yuan,J. and Horvitz,H.R. (1991) *Annu. Rev. Cell Biol.*, **7**, 663–698.

Enari,M., Hug,H. and Nagata,S. (1995) *Nature*, **375**, 78–81.

Erickson,S.L. *et al.* (1994) *Nature*, **372**, 560–563.

Farrow,S.N., White,J.H.M., Martinou,I., Raven,T., Pun,K.-T., Grinham, C.J., Martinou,J.-C. and Brown,R. (1995) *Nature*, **374**, 731–733.

Faucheu,C. *et al.* (1995) *EMBO J.*, **14**, 1914–1922.

Fernandes-Alnemri,T., Litwack,G. and Alnemri,E.S. (1994) *J. Biol. Chem.*, **269**, 30761–30764.

Fernandes-Alnemri,T., Litwack,G. and Alnemri,E.S. (1995) *Cancer Res.*, **55**, 2737–2742.

Gagliardini,V., Fernandez,P.-A., Lee,R.K.K., Drexler,H.C.A., Rotello, R.J., Fishman,M.C. and Yuan,J. (1994) *Science*, **263**, 826–828.

Gu,Y., Wu,J., Faucheu,C., Lalanne,J.-L., Diu,A., Livingston,D.J. and Su,M.S.-S. (1995) *EMBO J.*, **14**, 1923–1931.

Gulbins,E. *et al.* (1995) *Immunity*, **2**, 341–351.

Hartley,S.B., Cooke,M.P., Fulcher,D.A., Harris,A.W., Cory,S., Basten,A. and Goodnow,C.C. (1993) *Cell*, **72**, 325–335.

Hengartner,M.O. and Horvitz,H.R. (1994a) *Cell*, **76**, 665–676.

Hengartner,M.O. and Horvitz,H.R. (1994b) *Curr. Opin. Genet. Dev*, **4**, 581–586.

Herron,L.R., Eisenberg,R.A., Roper,E., Nakkanaiah,V.N., Cohen,P.L. and Kotzin,B.L. (1993) *J. Immunol.*, **151**, 3450–3459.

Hsu,H., Xiong,J. and Goeddel,D.V. (1995) *Cell*, **81**, 495–504.

Itoh,N. and Nagata,S. (1993) *J. Biol. Chem.*, **268**, 10932–10937.

Itoh,N., Yonehara,S., Ishii,A., Yonehara,M., Mizushima,S.-I.,

Samashima,M., Hase,A., Seta,Y. and Nagata,S. (1991) *Cell*, **65**, 233–243.

Itoh,N., Tsujimoto,Y. and Nagata,S. (1993) *J. Immunol.*, **151**, 621–627.

Jäättelä,M., Wissing,D., Bauer,P.A. and Li,G.C. (1995) *EMBO J.*, **11**, 3507–3512.

Jarvis,W.D., Kolesnick,R.N., Fornari,F.A., Traylor,R.S., Gewirtz,D.A. and Grant,S. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 73–77.

Ju,S.-T., Panka,D.J., Cui,H., Ettinger,R., El-Khatib,M., Sherr,D.H., Stanger,B.Z. and Marshak-Rothstein,A. (1995) *Nature*, **373**, 444–448.

Kägi,D., Vignaux,F., Ledermann,B., Burki,K., Depraetere,V., Nagata,S., Hengartner,H. and Golstein,P. (1994) *Science*, **265**, 528–530.

Kawabe,Y. and Ochi,A. (1991) *Nature*, **349**, 245–248.

Kiefer,M.C., Brauer,M.J., Powers,V.C., Wu,J.J., Umansky,S.R., Tomei,L.D. and Barr,P.J. (1995) *Nature*, **374**, 736–739.

Korsmeyer,S.J. (1995) *Trends Genet.*, **11**, 101–105.

Kuida,K., Lippke,J.A., Ku,G., Harding,M.W., Livingston,D.J., Su,M.S.S. and Flavell,R.A. (1995) *Science*, **267**, 2000–2003.

Kumar,S. (1995) *Trends Biochem. Sci.*, **20**, 198–202.

Kumar,S., Kinoshita,M., Noda,M., Copeland,N.G. and Jenkins,N.A. (1994) *Genes Dev.*, **8**, 1613–1626.

Lazebnik,Y.A., Kaufmann,S.H., Desnoyers,S., Poirier,G.G. and Earnshaw,W.C. (1994) *Nature*, **371**, 346–347.

Li,P. *et al.* (1995) *Cell*, **80**, 401–411.

Linette,G.P., Grusby,M.J., Hedrick,S.M., Hansen,T.H., Glimcher,L.H. and Korsmeyer,S.J. (1994) *Immunity*, **1**, 197–205.

Los,M. *et al.* (1995) *Nature*, **375**, 81–83.

Lowin,B., Hahne,M., Mattmann,C. and Tschopp,J. (1994) *Nature*, **370**, 650–652.

Lynch,D.H. *et al.* (1994) *Immunity*, **1**, 131–136.

Miura,M., Zhu,H., Rotello,R., Hartwieg,E.A. and Yuan,J. (1993) *Cell*, **75**, 653–660.

Mizushima,J. and Nagata,S. (1990) *Nucleic Acids Res.*, **18**, 5322.

Munday,N.A., Vaillancourt,J.P., Ali,A., Casano,F.J., Miller,D.K., Molineaux,S.M., Yamin,T.-T., Yu,V.L. and Nicholson,D.W. (1995) *J. Biol. Chem.*, **270**, 15870–15876.

Nicholson,D.W. *et al.* (1995) *Nature*, **376**, 37–43.

Nisitani,S., Tsubata,T., Murakami,M., Okamoto,M. and Honjo,T. (1993) *J. Exp. Med.*, **178**, 1247–1254.

Obeid,L.M., Linardic,C.M., Karolak,L.A. and Hannun,Y.A. (1993) *Science*, **259**, 1769–1771.

Oehm,A. *et al.* (1992) *J. Biol. Chem.*, **267**, 10709–10715.

Ogasawara,J., Watanabe-Fukunaga,R., Adachi,M., Matsuzawa,A., Kasugai,T., Kitamura,Y., Itoh,N., Suda,T. and Nagata,S. (1993) *Nature*, **364**, 806–809.

Oltvai,Z.N., Milliman,C.L. and Korsmeyer,S.J. (1993) *Cell*, **74**, 609–619.

Pezzella,F., Tse,A.G.D., Cordell,J.L., Pulford,K.A.F., Gatter,K.C. and Mason,D.Y. (1990) *Am. J. Pathol.*, **137**, 225–232.

Pfeffer,K. *et al.* (1993) *Cell*, **73**, 457–467.

Pullen,A.M., Marrack,P. and Kappler,J.W. (1988) *Nature*, **335**, 796–801.

Ray,C.A., Black,R.A., Kronheim,S.R., Greenstreet,G.S. and Pickup,D.J. (1992) *Cell*, **69**, 597–604.

Rothe,J. *et al.* (1993) *Nature*, **364**, 798–802.

Russell,J.H. and Wang,R. (1993) *Eur. J. Immunol.*, **23**, 2379–2382.

Russell,J.H., Rush,B., Weaver,C. and Wang,R. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 4409–13.

Scott,D.E., Kisch,W.J. and Steinberg,A.D. (1993) *J. Immunol.*, **150**, 664–672.

Sentman,C.L., Shutter,J.R., Hockenbery,D., Kanagawa,O. and Korsmeyer,S.J. (1991) *Cell*, **67**, 879–888.

Sidman,C.L., Marshall,J.D. and von Boehmer,H. (1992) *Eur. J. Immunol.*, **22**, 499–504.

Singer,G.G. and Abbas,A.K. (1994) *Immunity*, **1**, 365–371.

Stanger,B.Z., Leder,P., Lee,T.-H., Kim,E. and Seed,B. (1995) *Cell*, **81**, 513–523.

Strasser,A. (1995) *Curr. Opin. Immunol.*, **7**, 228–234.

Strasser,A., Harris,A.W. and Cory,S. (1991) *Cell*, **67**, 889–899.

Strasser,A., Harris,A.W., Von Boehmer,H. and Cory,S. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 1376–1380.

Suda,T., Takahashi,T., Golstein,P. and Nagata,S. (1993) *Cell*, **75**, 1–20.

Takahashi,T., Tanaka,M., Brannan,C.I., Jenkins,N.A., Copeland,N.G., Suda,T. and Nagata,S. (1994) *Cell*, **76**, 969–976.

Tartaglia,L.A., Ayres,T.M., Wong,G.H.W. and Goeddel,D.V. (1993) *Cell*, **74**, 845–853.

Tewari,M. and Dixit,V.M. (1995) *J. Biol. Chem.*, **270**, 3255–3260.

Thornberry,N.A. *et al.* (1992) *Nature*, **356**, 768–774.

Trauth,B.C., Klas,C., Peters,A.M.J., Matzku,S., Moller,P., Falk,W., Debatin,K.-M. and Krammer,P.H. (1989) *Science*, **245**, 301–305.

Vandenabeele,P., Declercq,W., Vanhaesebroeck,B., Grooten,J. and Fiers,W. (1995) *J. Immunol.*, **154**, 2904–2913.

Vanhaesebroeck,B., Reed,J.C., de Valck,D., Grooten,J., Miyashita,T., Tanaka,S., Beyaert,R., van Roy,F. and Fiers,W. (1993) *Oncogene*, **8**, 1075–1081.

Vaux,D.L., Cory,S. and Adams,J.M. (1988) *Nature*, **335**, 440–442.

Vaux,D.L., Weissman,I.L. and Kim,S.K. (1992) *Science*, **258**, 1955–1957.

Wang,L., Miura,M., Bergeron,L., Zhu,H. and Yuan,J. (1994) *Cell*, **78**, 739–750.

Watanabe-Fukunaga,R., Brannan,C.I., Copeland,N.G., Jenkins,N.A. and Nagata,S. (1992) *Nature*, **356**, 314–317.

Wyllie,A.H., Kerr,J.F.R. and Currie,A.R. (1980) *Int. Rev. Cytol.*, **68**, 251–306.

Yang,E., Zha,J., Jockel,J., Boise,L.H., Thompson,C.B. and Korsmeyer, S.J. (1995) *Cell*, **80**, 285–291.

Yonehara,S., Ishii,A. and Yonehara,M. (1989) *J. Exp. Med.*, **169**, 1747–1756.

Yuan,J., Shaham,S., Ledoux,S., Ellis,H.M. and Horvitz,H.R. (1993) *Cell*, **75**, 641–652.